

# Thermal Inactivation of Newcastle Disease Virus<sup>1</sup>

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The rate of destruction of hemagglutinins and infectivity of Newcastle disease virus was determined over a temperature range of 37.8 to 60 C. From the calculated values of  $\Delta H$  and  $\Delta S$ , it was concluded that inactivation of the hemagglutinating activity and viral infectivity was due to protein denaturation.

The significance of virus-contaminated foods in human diets is not yet established. However, food, including milk, has been implicated epidemiologically with several outbreaks of viral diseases (1, 6, 7, 8, 12, 17). Poliovirus and echovirus have been isolated from the soil of fields irrigated with sewage, and a few vegetables grown in these fields have been shown to contain cytopathic agents (4). Although it is known that viruses do not replicate in foods, echovirus, coxsackievirus, and poliovirus have been shown to survive on vegetables stored under household conditions for as long as 2 months (5). These findings seem to indicate that viruses pathogenic to man can gain entrance to foods during their production, processing, or preparation. Food processes designed for the elimination of harmful organisms from food cannot be assumed to eliminate foodborne viruses (13).

Thus, it becomes a matter of practical importance to determine the thermal resistance of viruses. It is also of interest to study the kinetics of the thermal inactivation, since it may give some insight to the mechanism of inactivation. The present study reports experiments measuring energy of activation and entropy of activation of the thermal inactivation of hemagglutinin and infectivity properties of Newcastle disease virus (NDV).

## MATERIAL AND METHODS

**Virus.** NDV-cal. was obtained from the Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass. Allantoic fluid harvested 48 to 72 hr after infection of 10-day-old embryos was used as virus-containing material. The sterile fluids possessing a hemagglutinin titer of 1,280 or higher were pooled, centrifuged at 8,500 rev/min

for 20 min, and stored at  $-40^{\circ}\text{C}$  in 15-ml portions until used.

**Hemagglutinin titration.** The virus in 0.5-ml amounts was serially diluted by factors of two in test tubes containing immunological buffer ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 27.6 g;  $\text{NaCl}$ , 29.25 g;  $\text{NaOH}$ , 6.05 g/liter). To each tube of virus, a 0.5-ml quantity of a 0.25% suspension of chicken red blood cells in immunological buffer was added. The virus titer was read after 1 hr at room temperature. The titer was determined from the highest dilution of virus which provided a visible pattern of agglutination. Agglutination patterns were read as + + + +, + + +, + +, +, 0 and were expressed as hemagglutinating units per ml.

**Virus infectivity titration.** Tenfold dilutions of virus samples were made in phosphate-buffered saline containing 1% rabbit serum. One-tenth milliliter amounts of each dilution were injected into the allantoic cavity of six 10-day-old fertile hens' eggs and incubated for 1 to 5 days. Each day the eggs were candled, and the dilutions at which embryos died were recorded. The end point was taken as the highest dilution which resulted in 50% mortality of embryos. The 50% lethal end point ( $\text{LD}_{50}$ ) was determined by plotting the logarithm of the per cent mortality of the dilution just above 50% and just below 50% mortality versus the dilution of virus. The curve was drawn through these two points, and the point where the curve intersected the 50% survival line was noted. The reciprocal of this value was regarded as the  $\text{LD}_{50}$  per 0.1 ml.

**Thermal inactivation studies.** A series of glass tubes (8 mm in diameter by 150 mm in length) containing 3-ml amounts of diluted infective allantoic fluid (pH 7.7) were heated for various times in a thermostatically controlled water bath which was constant to  $0.1^{\circ}\text{C}$  and were then immediately cooled in ice water and assayed for hemagglutinins and infectivity by the methods described previously.

## RESULTS

**Inactivation of hemagglutinin.** The inactivation curves obtained for NDV hemagglutinin when allantoic fluids containing virus were heated at four different temperatures, ranging from 43.3 to 60 C, are shown in Fig. 1. The thermal inac-

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tivation curves are considered linear over the temperature region studied, indicating first-order kinetics. There was no reduction in hemagglutinin activity after 75 min at 43.3 C, but at the higher temperatures there was a loss which was a function of temperature.

The velocity constant ( $k$ ) for the inactivation at a given temperature was calculated from the equation  $k = (2.303 \log V/V_0)/t$ , where  $V_0$  is the initial activity and  $V$  is the activity after heating for time  $t$ .

The temperature dependence of the hemagglutinin inactivation reaction, as indicated by an Arrhenius plot, is presented in Fig. 2. The velocity constants for the inactivation of hemagglutinin at various temperatures have been plotted as a function of the reciprocal of the absolute temperature ( $1/T$ ). Although the three experimental points did not fall on a straight line, it was assumed that a linear relation existed, and a regression line was drawn by the method of least squares.

The energy of activation,  $\Delta H$ , and the entropy of activation,  $\Delta S$ , were calculated from Eyring's theory of absolute reaction rates by use of the identity  $1n k = 1n (KT/h) - (\Delta H/RT) + (\Delta S/R)$ , where  $K$  is Boltzmann's constant ( $1.38 \times 10^{-16}$  ergs/deg),  $T$  is the absolute temperature,  $h$  is

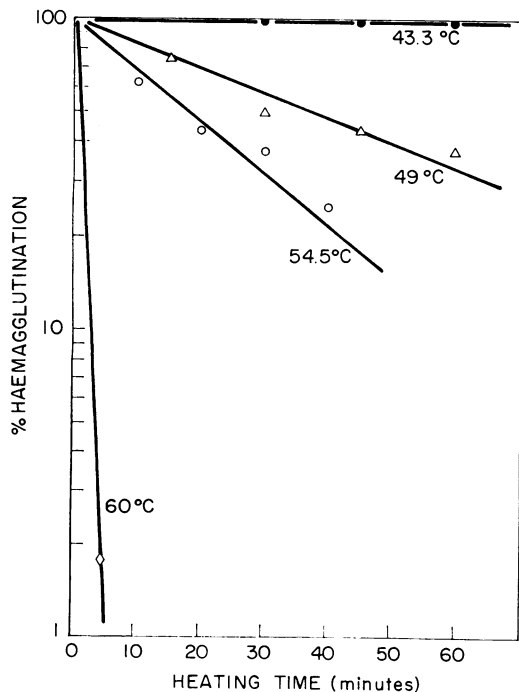


FIG. 1. Rate of inactivation of hemagglutinin activity of Newcastle disease virus at various temperatures.

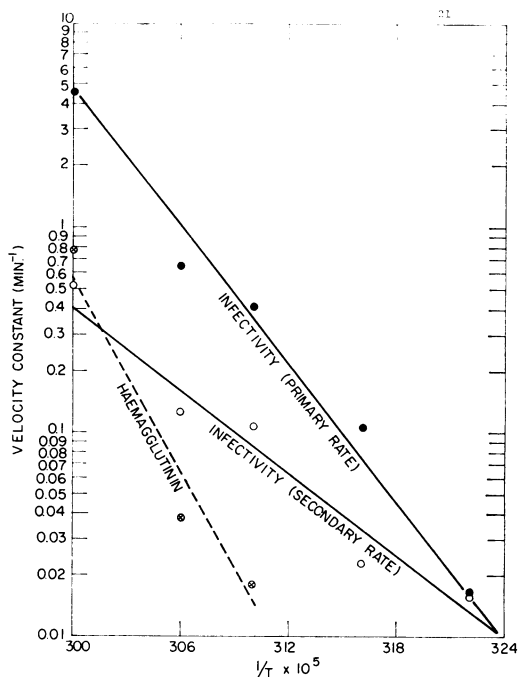


FIG. 2. Temperature dependence of the thermal inactivation rate constants for hemagglutinin activity and infectivity of Newcastle disease virus.

Planck's constant ( $6.625 \times 10^{-27}$  erg-sec), and  $R$  is the gas constant (1.987).  $\Delta H$  was determined by solving simultaneous equations for two values of  $k$  at two different temperatures and then substituting this value back to obtain  $\Delta S$ . These thermodynamic parameters are listed in Table 1.

**Inactivation of infectivity.** The rates of inactivation of NDV infectivity at temperatures ranging from 37.8 to 60 C (100 to 140 F) are shown in Fig. 3. The curves for inactivation at 37.8 and 43.3 C were constructed by means of regression analysis, and for the remaining heating temperatures the curves were fitted by eye. It is evident that at temperatures above 37.8 C the thermal inactivation of NDV infectivity took place in a two-component fashion, each following first-order kinetics. This type of thermal inactivation curve is representative of many viruses.

An Arrhenius plot of the velocity constants is also presented in Fig. 2 for both the primary inactivation rates (fast-inactivating component) and the secondary inactivation rates (slow-inactivating component). The best straight line through each set of data points was drawn by regression analysis. The two straight lines thus obtained converged at an absolute temperature of approximately 309 K (36 C). The energy of activation and entropy of activation were calcu-

TABLE 1. *Energy of activation and entropy of activation for inactivation of Newcastle disease virus hemagglutinating and infectivity properties*

Property	Temp range (C)	$\Delta H$ (cal/mole)	$\Delta S$ (cal per mole per degree)
Hemagglutinin.....	49–60	70,500	143.4
Infectivity (primary inactivation rate)....	37.8–60	52,900	95
Infectivity (secondary inactivation rate)....	37.8–60	30,900	22.4

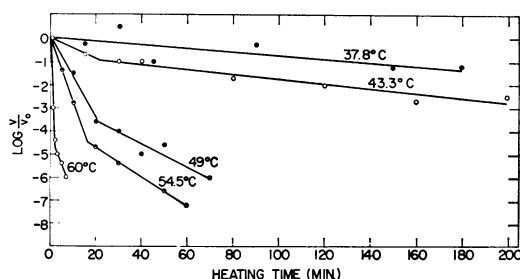


FIG. 3. *Rate of destruction of infectivity of Newcastle disease virus at various temperatures.*

lated as previously described, and the values are listed in Table 1. The calculated value of activation energy for inactivation of NDV infectivity falls within the range cited for denaturation of various proteins (18).

## DISCUSSION

NDV is a ribonucleic acid (RNA) virus containing an inner coiled helix of ribonucleoprotein surrounded by an outer protein envelope. In addition, the virus contains some lipid material. The hemagglutination properties of the virus are associated principally with the protein of the outer coat. Denaturation of this outer protein should then result in a loss of hemagglutinin activity. The large values of  $\Delta H$  (70.5 kcal/mole) and  $\Delta S$  (143.4 cal per mole per degree) obtained in this study for inactivation of hemagglutinin activity are compatible with the requirement for protein denaturation (18).

The two-component curve which characterized the thermal inactivation of NDV infectivity has been reported for other viruses (3, 15, 16, 19). The shape of these curves has been attributed by some to an inherent heterogeneity of the virus, such that a small proportion of the thermally resistant particles account for the decreased slope of the latter segment of these curves. However,

in the present study, it would appear that the fraction of resistant virus, which can be estimated by extrapolating the secondary portion of the inactivation curve to the ordinate, is not some fixed value but is dependent on the heating temperature and disappears at low temperatures. It is difficult to reconcile this result with the theory of a heterogeneous resistance. Others have postulated that the change in inactivation rate at high temperature is due to factors operating during the reaction, such as the formation of aggregates, adsorption to the walls of the vessel, or the presence of virus particles in aerosol droplets above the surface of the liquid (14), all of which may protect against inactivation. Polioviruses being treated with formaldehyde become progressively more resistant to inactivation by this compound, and it has been suggested that this is due to a hardening of the protein coat as the reaction proceeds (10). Nevertheless, it appears that the two-component inactivation curves for viruses will have to be accepted until the question is resolved as to whether this phenomenon is due to an artifact or to a particular inactivation mechanism.

Inactivation of NDV infectivity can be caused by destruction of the RNA, denaturation of the nucleoprotein, or denaturation of the protein in the outer coat resulting in an inability of the virus to attach to host cells.

Denaturation of protein is associated with a large value of  $\Delta H$  and  $\Delta S$  due to the rupture of a large number of hydrogen bonds which results in a collapse or unfolding of the secondary structure of the molecule. Smaller values of  $\Delta H$  and  $\Delta S$  are required for heat inactivation of viral RNA. Values of about 20 kcal/mole for  $\Delta H$  and  $-19$  cal per mole per degree for  $\Delta S$  for thermal inactivation of tobacco mosaic virus RNA and 31 kcal/mole for  $\Delta H$  and 4 cal per mole per degree for  $\Delta S$  for thermal inactivation of a poliovirus RNA have been reported (9, 11). Ginoza (11) postulated that inactivation of RNA is due to a rupture of the chain. The rather large values of  $\Delta H$  and  $\Delta S$  obtained in the present study for inactivation of NDV infectivity would indicate that loss of infectivity in the high temperature range was due to protein denaturation rather than destruction of RNA. In support of this conclusion is the fact that a poliovirus, a rhinovirus, and foot-and-mouth disease virus heated at high temperature (50 to 65 C) show a marked reduction in viral infectivity but only a slight loss in infectivity of the extracted RNA (2, 9). It should be pointed out that it has been found with some viruses, at least, that at low heating temperatures (about 43 C or less) loss of infectivity is due to inactivation of RNA (9).

It appears that this phenomenon may also apply to NDV. Note that there was no damage to protein (hemagglutinin) after heating for 75 min at 43.3 C (Fig. 1), whereas this same time-temperature treatment caused over 90% loss in infectivity (Fig. 2).

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